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THE DEVELOPMENT AND SYSTEMATIC POSITION OF ARACHNIOTUS TRISPORUS[1]

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INTRODUCTION

In 1937 De Lamater described crozier formation in a species of *Arachniotus*, this being the first publication of such a phenomenon in the Gymnoascaceae. The species with which he was working was very similar to *A. aureus*. A year earlier Hotson, in Seattle, Washington, had described a new species of *Arachniotus*, *A. trisporus*, while also in 1936 Vailionis, at Kaunas, Lithuania, described what he thought was a new species, naming it *Gymnoascus sudans*. However, when Vailionis' culture reached the Centraalbureau voor Schimmelcultures, at Baarn, Netherlands, it was identified as *A. trisporus* Hotson. In order to learn whether this species has the same crozier formation as the species of *Arachniotus* with which De Lamater worked we obtained a subculture of Vailionis' strain from Baarn. Upon finding that croziers were produced, a cytological study was made of the organism, as well as a study to determine as clearly as possible its systematic position and its true identity. The results are recorded in the present paper.

HISTORY

The genus *Arachniotus* was created by Schroeter in 1893 to include three species, *A. ruber*, *A. candidus*, and *A. aureus*, the first having been described by Van Tieghem in 1877 as *Gymnoascus ruber* and the other two by Eidam in 1886 as *G. candidus* and *G. aureus*. Schroeter described this new genus as having a globose fruit-body with spherical or ellipsoidal spores, the membranes of which are hyaline, golden, or red. The peridium, the characteristics of which separate this genus from *Gymnoascus*, is composed of uniform hyphae interwoven so as to form a web-like membrane.

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[1] An investigation carried out in the graduate laboratory of the Henry Shaw School of Botany of Washington University and submitted as a thesis in partial fulfillment of the requirements for the degree of master of science in the Henry Shaw School of Botany of Washington University.

Van Tieghem ('77) found *A. ruber* on the dung of the rat and dog. Schroeter ('93) reported finding it on the dung of the dog and goat at Breslau, Germany, and since then it has been found (Massee and Salmon, '02) on cat's dung from Aburi, Gold Coast of Africa. This species differs from the others in having an orange-red or red fruit-body.

Eidam ('86) first found *A. candidus* in Breslau on boiled rice on which he was cultivating *Aspergillus fumigatus*. Schroeter ('93) reported it upon owl's dung in Brieg, Germany. It has also been found (Massee and Salmon, '02) on an old nest of a wild bee and on dung of the common roe, both at Kew, England. The persistent snow-white fruit body and the smooth ascospores are its chief characteristics.

Eidam makes no mention of where he found *A. aureus*, but reports that he repeatedly cultivated it on bread and paper. Schroeter ('93), however, states it was found on decaying vegetables. According to him, it differs from *A. candidus* chiefly by its golden-yellow, minutely spiny ascospores. Another important characteristic is the presence of hyphae in the form of fine spirals.

Massee and Salmon ('02) described a new species, *A. citrinus*, found on the dung of the giant kangaroo in Kew, England. This species resembles *A. aureus* to a great extent, but the color is lemon-yellow rather than golden-yellow, and its ascospores are smooth rather than rough.

Shear, in 1902, described a new species which he called *A. trachyspermus*, associated with diseased cranberries in New Jersey. Like *A. candidus*, it has a white peridium, but its ascospores are echinulate-roughened and show a faint greenish-yellow tint.

In 1936 Hotson found *A. trisporus* in contaminated milk, and Vailionis found the same species (his *Gymnoascus sudans*) as a contamination in a nutrient solution in which he was growing some small branches of birch. According to Hotson, *A. trisporus* differs from other species of genus in the size of the fruit-body, the smooth ascospores, and the three types of spores in its life cycle—ascospores, conidiospores, and chlamydospores. Although the fruit-body is smaller than that reported for *A. candidus*, the two species are very similar, both having smooth ascospores and three types of spores in their life cycle.

MATERIALS AND METHODS

As has been stated, we obtained Vailionis' strain of *Arachniotus trisporus* Hotson from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands, in order to compare it with a known species. This known species was obtained from De Lamater, being a subculture of one which he had received from Baarn and identified by Nannizzi as *A. aureus*. Also, a culture was received from Hotson of his strain of *A. trisporus*.

For general study of the organism, slides were made with Maneval's lactophenol (Maneval, '36) every two or three hours after germination until the time that the ascospores were fully developed from both agar and broth media. For

cytological studies the organism was imbedded in paraffin and stained with Haidenhain's iron-haematoxylin.

For cytological study agar slants in test-tubes proved to be the most adaptable of media. By inoculating many tubes at the same time with spores suspended in sterile distilled water and making slides with lacto-phenol of one tube at regular intervals, it was possible to determine whether the culture was in the desired phase. Killing was accomplished very easily by pouring Gilson's fluid into the test-tube. Flemming's weaker solution was also used, but was not nearly so good as Gilson's for this type of material. The tube containing the fixed material was placed in a water bath into which tap-water was continuously running. The agar slant was removed afterward by gently shaking.

The following schedule was followed for dehydrating:

Gilson's fluid	24–48 hrs.
Washing	24 hrs.
5, 10, 20, 35, 50, 70% alcohol	30 min. each
85% alcohol containing a few drops of iodine[1]	2 hrs.
95% alcohol	30 min.
100% alcohol	Overnight
5, 15, 25, 50, 75% xylol in absolute alcohol	30 min. each
100% xylol	Overnight

Imbedded in paraffin 3 days

Sections of 10 μ thickness were quite satisfactory.

The following schedule was followed for staining:

100, 66, 33% xylol	5 min. each
100, 95, 85, 70, 50, 35% alcohol	5 min. each
Wash 10 times over a period of 20 minutes	
4% iron alum	2 hrs.
Wash as before	
½ or ¼% haematoxylin	Overnight
Differentiate in 4% iron alum	1–2 mins.
(5 seconds makes a difference)	
Wash as before	
35, 50, 70, 85, 95, 100% alcohol	5 min. each
50, 100% xylol	5 min. each

Mount in balsam

Sections were usually counterstained overnight in safranin, erythrosin, or phloxine, dissolved in 50, 70, and 95 per cent alcohol, respectively. A .01 per cent solution of fast green was dissolved in absolute alcohol, and the material immersed in this for one minute only. Germination of spores was studied from slides made with lacto-phenol from spore dilutions at various intervals. For colony studies Petri dishes were inoculated with single spore cultures obtained from making dilution plates of spores. These were incubated, and the colonies measured at regular intervals.

THE ORGANISM

Germination of spores.—The spores of *A. trisporus* germinate usually between 24 and 36 hours after inoculation. The chlamydospores seem to germinate more

[1] Just enough iodine to color is added to prevent precipitation of the mercuric chloride in the material (Kingsbury and Johannsen, '27).

readily than conidia or ascospores. In the process of germination the spore swells to about twice its normal size and at the same time one end of the wall becomes very thin. Soon the wall breaks and the germ-tube appears (pl. 2, fig. 21a, b, f, g). In the ascospore, the tube is produced almost invariably at either of the narrow ends (fig. 21g), and in the chlamydospore at either the blunt or apical end (fig. 21b). When the spore has more than two germ-tubes, they are produced opposite each other as is shown in fig. 21d. The germ-tube, upon lengthening, begins to branch (fig. 21e), but it is still non-septate, except for one septum laid down close to the original spore. After 8 to 10 hours, these branches usually produce immature chlamydospores and conidia which are soon cut off by septa.

Vegetative mycelium.—As Vailionis ('36) has already reported, this organism forms two layers when grown in a liquid nutrient medium, the lower layer being the vegetative mycelium, and the upper the reproductive mycelium. The lower layer is usually imbedded in the medium, whether the latter be a liquid or a solid. If the inoculum is a spore dilution the spores germinate in the liquid and grow immersed for about 72 hours (measuring from the time of inoculation). At about this time the mycelium rises to a position just below the surface where it begins to produce a white compact but fluffy upper layer. The time for the production of this layer varies according to the type of inoculum and the nutrients employed in the medium. If the inoculum is a bit of mycelium, the latter usually floats on the surface, producing the two layers simultaneously. No general statement can be made concerning the nutrients or percentage of nutrients most favorable to growth except that there is great variation. In a solid medium, both layers develop simultaneously, but it is to be noted that the lower layer does not develop as extensively as in a liquid medium, probably due to a lower oxygen tension.

The lower layer consists of simply branched hyphae. The length of the cells may vary, roughly, between 10 and 40 μ, but most frequently between 15 and 20 μ. Vailionis found that the length was about 44 μ. The width of hyphae seems to be more constant than the length. This is usually between 1.0 and 3 μ, although Vailionis found it to be 7.9μ. The thickness of the hyphae does not necessarily decrease with increase in length. Usually hyphae that increase in length also increase in width and vice versa.

The very young hyphae, i. e., those at 36 to 49 hours (from the time of inoculation), are densely filled with cytoplasm. Vacuoles appear at about 48 hours (pl. 1, fig. 1). Septa, however, usually appear before vacuoles, which become larger and more distinct in ten hours (fig. 2). At about 70 hours more vacuoles have appeared, but they are smaller than previous ones (fig. 3). By 80 hours (fig. 4), the cells have become so vacuolate that the cytoplasm appears to be a fine net and septa are very hard to distinguish although they are most likely still present. In ten more hours the fine reticulation of the cells has been replaced by a very loose network (fig. 5). By the time that ascospores are fully formed, 144 hours, these cells are dead. It can then be said that the vegetative

hyphae pass gradually from a state of dense cytoplasm with definite vacuoles to one of little cytoplasm with indefinite vacuoles.

When the culture is from one to two weeks old, an exudate is produced on top of the mycelium, in the form of small colorless droplets which collect later to form large yellow drops. Vailionis noted this exudate and thought it appropriate to name his new species *Gymnoascus sudans*. From observing the condition of old hyphae and the production of this exudate, the writer has concluded that the latter comes from the vacuoles after the disintegration of old hyphae. De Lamater observed hyphal fusions in his species of *Arachniotus*, but no such phenomenon has been observed in *A. trisporus*.

The vegetative cells are always multinucleate. There does not seem to be any definite number, order, or arrangement (pl. 1, figs. 8-12). Even the size of the nuclei varies as is illustrated. Sometimes it appears, especially in hyphae 48 to 72 hours old, that the nuclei are paired and surrounded by a definite vacuole, but there are so many exceptions that it cannot be said to be a general rule. The writer has not observed any division of nuclei or mitotic figures in the vegetative cells. The nuclei of these hyphae degenerate with the cytoplasm of the cells as is illustrated in pl. 1, fig. 12.

Racquet mycelium.—Racquet mycelium is found in this species as in other species of *Arachniotus*. The cells composing this mycelium are greatly swollen at one end, appearing as clubs attached end to end (pl. 2, figs. 13, 14). They may be greatly distorted as in fig. 13 or hardly discernible from the rest of the hyphae. Hotson ('36) believes that these cells may be food-storage organs. Vailionis ('36) noted them as a characteristic of his species but did not suggest any particular function for them. Nannizzi ('26) pointed out the relationship of the Dermatophytes with *Arachniotus candidus* and with other closely related genera through the presence of this racquet mycelium, as well as other characteristics.

The writer has noted racquet mycelium on all types of nutrients, both solid and liquid. The three cultures of *Arachniotus* were grown on a medium consisting of agar and distilled water in order to see if this type of hypha was produced. It did not appear in either Vailionis' or Hotson's strain of *Arachniotus trisporus*. A few club-shaped cells, however, were observed in Nannizzi's culture, which would seem to indicate that this type of mycelium has nothing to do with food storage as Hotson thought.

Some cells are comparatively short (20 μ) for their width while some may be as long as 60 to 70 μ. Generally, the width varies between 8 and 20 μ. The swelling is usually greater on one side than on the other, and one end is always more densely filled with cytoplasm than the other. Sometimes the cytoplasm shrinks away from the cell wall, causing the latter to wrinkle (pl. 2, fig. 14). Since the racquet mycelium is purely vegetative, it degenerates during the sexual development and is hardly discernible when the ascospores are mature. It can be

concluded from these observations that a racquet mycelium is characteristic of the three strains of *Arachniotus* studied.

Chlamydospores.—The chlamydospores are produced in the upper part of the vegetative layer, as Vailionis ('36) indicates, terminating the ends of short branches. Vailionis reports that they are 8.8 μ in diameter, but the writer has found that not only do they vary in size but also that they are generally pyriform. The width may range from 4.4 to 8.7 μ, but most often from 6 to 8 μ. The length may be as little as 4.4 μ and as much as 11.6 μ, but 8 to 10 μ covers the range in which most of them are found.

The chlamydospore is the first spore to make its appearance in the development of *A. trisporus*, beginning to form almost immediately after the germ-tube has lengthened. Each one is produced on the end of a short branch consisting of one cell, which increases in length as the spore matures (pl. 2, fig. 15). Sometimes, this branch may produce another side-branch below the spore, in which case a cell wall is laid down between the spore and the side branch. The immature spores are non-vacuolate, but as they mature a vacuole makes its appearance (fig. 15b), followed by others. At this time if conditions are favorable (42 to 69 hours) the chlamydospores, instead of forming a heavy cell wall, form an extension of protoplasm at the apical end of the spore (pl. 2, fig. 17). This new growth, a continuation of the hypha on the other end of the cell, results in an intercalary chlamydospore, already noted by Vailionis. It is not uncommon to see that the chlamydospore has sprouted in two places (fig. 16). The hypha produced in such a manner is normal in all respects and may grow to a great length. Sometimes two chlamydospores are found on the same branch separated by only one cell. The occurrence of such a hypha can be explained by the fact that it was produced from the lower chlamydospore and that it in turn produced the terminal chlamydospore.

The several vacuoles in the chlamydospores fuse to form a central one after about 72 hours. The cell wall also begins to thicken at this time (pl. 2, fig. 18). The spore now has usually reached its maximum size and may remain at this size or shrink slightly. As the cell wall becomes thicker, the spore assumes a color between a cream and a cartridge-buff, and may round up as is shown in fig. 19b. The hypha slowly disintegrates, leaving the chlamydospore free.

An examination of the cultures stained with haematoxylin shows that the spores are multinucleate. The number of nuclei is indefinite, and it may vary as much as do those in the cells of the hyphae. The nuclei are situated in the cytoplasm close to the cell wall (pl. 2, fig. 20).

It is evident that the chlamydospores in this organism are produced as an insurance against unfavorable conditions, since they occur only at the beginning of the life cycle and since a large umber are produced especially under unfavorable conditions. Hotson ('36) found that the chlamydospores were the only ones to germinate after a period of time. He discovered, in 1925, that it was possible to

obtain transfers from cultures eight years old. By use of Van Teighem cells, he found that the only spores which germinated were chlamydospores. In 1936, he obtained the same results, the original culture being then twenty years old. It has not been possible to test Vailionis' culture but most likely, since the two cultures are quite similar, the results would be the same.

Conidia.—Conidia soon appear in the upper or reproductive layer, after chlamydospores have already begun to form. They are cut off exogenously from a phialide in a manner very similar to that in which conidia are cut off in *Aspergillus* (Thom and Church, '26) and in *Penicillium* (Thom, '30). The phialide is bottle-shaped with a long narrow neck. When a conidium begins to form the apex of the neck swells into a small pyriform, hyaline spore. This is cut off by a cell wall but remains attached to the phialide by the thin and almost invisible cell wall of the latter (pl. 3, fig. 23). A second conidium is formed below the first, and a third below the second, etc., making the first conidium the terminal cell. After the spore has been cut off from the phialide, it continues to grow so that the terminal one is the largest and the others are relatively smaller. The spores are at first densely filled with cytoplasm but as they age a vacuole appears which usually occupies most of the cell (fig. 25). Upon aging the conidium, unlike the chlamydospore, does not secrete a heavy wall but assumes the color of the chlamydospore. The writer's conclusion is that the color of old cultures is due to the color of conidia and of chlamydospores as well as the exudate.

The phialides usually arise in pairs from a cell which may come directly from the vegetative hypha (pl. 3, fig. 27b), or a specialized branch which bears other cells of this type (fig. 27a). Branching is usually dichotomous. This type of conidiophore is strikingly similar to some of those which are produced in *Penicillium*. A comparison of these illustrations with those of Thom ('30) bears out this point. Vacuolation of phialides is relatively constant, and the same for the three strains studied (fig. 23).

The size of conidia is not a constant character, nor could it be considered one as it depends upon their place in the chain, as has already been noted. They may be from 1.5 to 7.3 μ in length and from 0.7 to 4.4 μ in width, but those most commonly observed were 1.5 to 1.9 μ in width and 2.2 to 3.1 μ in length. Vailionis found them to be 5.3 μ in length and 4.4 μ in width.

Vailionis reports that conidia are produced under favorable conditions, but the writer has found that usually they are produced in abundance on agar in which there is no nutrient. They appear in about 18 hours after spore germination and are present in cultures thereafter. Figure 22 of pl. 3 shows a branch of a hypha developing into phialides. Conidia, however, are produced in abundance until copulation begins, a period of 24 hours in normal cultures. Phialides are less in evidence after 78 hours and have disappeared by the time of ascus formation. It is interesting to note that some of the phialides, after producing the spores, proliferate as a regular hypha (fig. 25). This phenomenon has occurred in

Sterigmatocystis auricoma described by Guegúen ('99) and noted by Thom and Church ('26). In this species the secondary sterigmata developed into short hyphae.

An unusual type of phialide and conidium was found on *A. trisporus* produced on oat-meal agar between 60 and 69 hours after inoculation. It occurred only once, and attempts to achieve the same results again were unsuccessful. The phialides were grouped together in such a fashion that from three to five appeared to have arisen from the end of a hypha (pl. 3, fig. 24). Closer inspection showed that the cells supporting the phialides were much reduced, appearing somewhat as in *Penicillium* sect. *Monoverticillata*. Adjacent to these unusual structures was found the common type of conidia as shown in fig. 27. The conidia were much smaller than the ordinary, being 1.3 μ in length.

It is difficult to determine the number of nuclei in one conidium. The writer has seen spores stained with haematoxylin that show both one and two nuclei in the cell (pl. 3, fig. 26). Whether this is the true state or not could not be determined. It is possible that one nucleus in the apparently uninucleate cells did not stain or that one "nucleus" in the binucleate cells was an artifact.

It is evident from this study that the production of conidia greatly facilitates the spread of this fungus. They are produced in great abundance around the margins of colonies upon aerial hyphae. Theoretically, by this means of reproduction the colony produced from a single spore has the potentiality of continuing growth so long as there is a suitable substratum. The writer has noticed this production of conidia both in a liquid and solid medium.

SEXUAL REPRODUCTION

As the production of conidia declines, sexual reproduction begins. The colony continues reproducing asexually around its margins, while in the center the sexual phase develops. Both Nannizzi ('26) and De Lamater ('37) noted a color change in the mycelium of their species upon the advent of the sexual phase, but we found that *A. trisporus* remains pure white as before. De Lamater also noted that cultures kept in the dark fruit most readily. To test this, six tubes of Sabouraud's agar were inoculated with spores of *A. trisporus*, three placed in the dark and three in the light, and all incubated at approximately the same temperature. It was found that the three in the light developed hyphae from 24 to 48 hours later than those in the dark. After the spores had germinated in the light-exposed tubes, sexuality developed in the normal length of time.

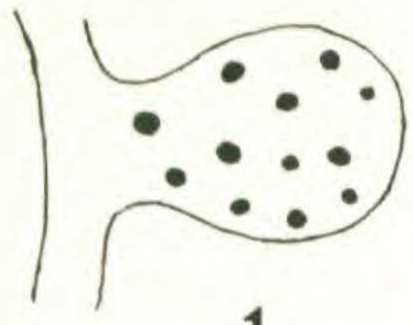
Copulation branches.—The first signs of sexuality are the production of the copulation branches (pl. 4, fig. 28). Baranetzky ('72), who first described *Gymnoascus Reessii* in any detail, a species very similar in development to *Arachniotus trisporus*, designated the male copulation branch as the "sterile cell" and the female as the "ascogene," the latter term from the fact that the branch develops ascogenous hyphae. These arise close to one another, evidently from

vegetative hyphae, but unlike them, are densely filled with cytoplasm. These two copulation branches are not identical in appearance, as were those De Lamater found in his species of *Arachniotus*, but are easily distinguishable by certain characters: (1) The female copulation branch is always longer than the male; (2) it usually becomes septate but the septation is not always evident; (3) the female or terminal cell, which stains very dark, is produced on the end of a fairly long branch (figs. 28b, d, f, and h), while the male cell, which stains just as dark, is produced as a short subterminal branch (figs. 28a, c, e, g). Frequently the male and female copulation branches arise adjacent to each other on the same hypha (fig. 28f and g) but they may be formed separately. Dale ('03) and De Lamater ('37) report the same conditions. Dale, who was working with *Arachniotus candidus*, states that the male branch is first formed and that the female grows around it afterward. In *A. trisporus*, however, the two copulation branches arise simultaneously (figs. 28f, g).

Copulation and fertilization.—The immature copulation branches are not cut off by a septum from the hypha (pl. 4, figs. 28e, f, g). At this time the male and female copulation branches begin to attract each other. Since the female branch is much longer than the male, it is able to draw or to grow towards a male branch and begin to coil around it (fig. 29), whereas the short male branch must remain in its original position. De Lamater states that in his species the two similar branches lying adjacent to each other elongate, swell, and coil up together. It seems that where the two copulation branches are borne close together on the same hypha (figs. 28f, g, and fig. 32) the tendency is for them to repel one another and be attracted by the opposite sex on another hypha. A septum now appears on both branches separating the cells containing the female and male nuclei from the rest of the hyphae (fig. 30). The female copulation branch which has already begun to encircle the male branch forms a tight coil of two or three turns (figs. 30, 31). Several septa have now appeared on the female branch, all formed below the first one (fig. 30). The terminal cell elongates but does not divide. It is this cell which lies adjacent to the male branch in the coil. The male branch elongates but remains unicellular (figs. 31-33). Both the terminal male and female cells are multinucleate (text-figs. 1-3).

Although the writer has not seen the actual movement of the male nuclei to the female branch, she is certain that this must occur. As has been stated, the male copulation cell contains many nuclei which are spaced irregularly. The terminal cell of the female branch is also multinucleate but the nuclei are spaced evenly. Some time later, after the tip of the female branch has come in contact with the male cell (pl. 4, fig. 31), it is found that there are pairs of nuclei evenly spaced in the female branch and that the male branch contains either few or no nuclei. From these observations the writer concludes that the male nuclei migrate into the female branch and pair with the female nuclei.

In this species, copulation and fertilization are much as reported by Dale



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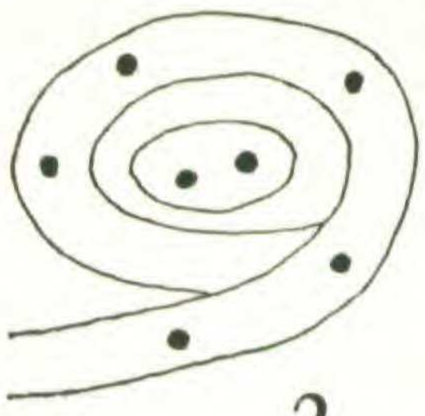

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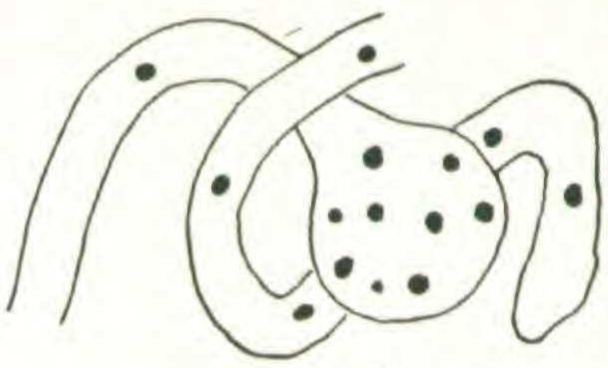

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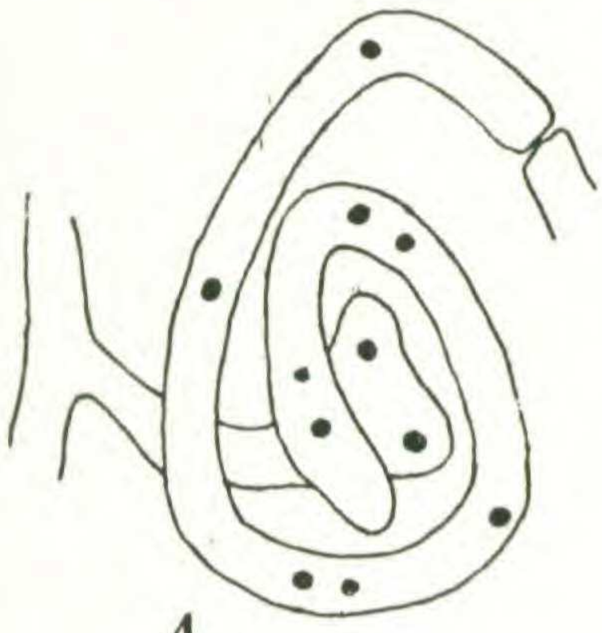

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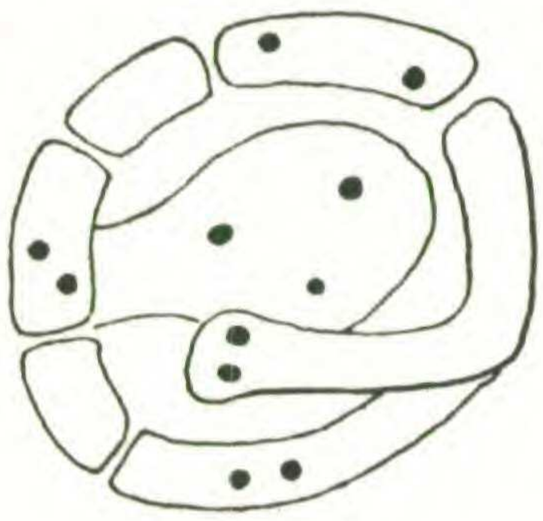

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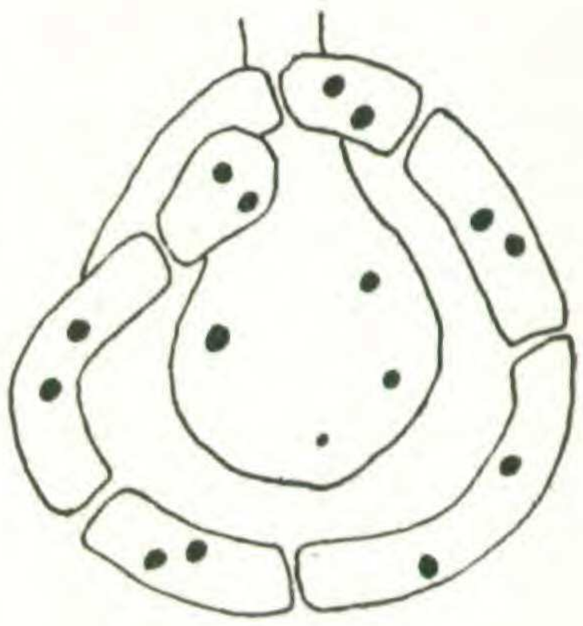

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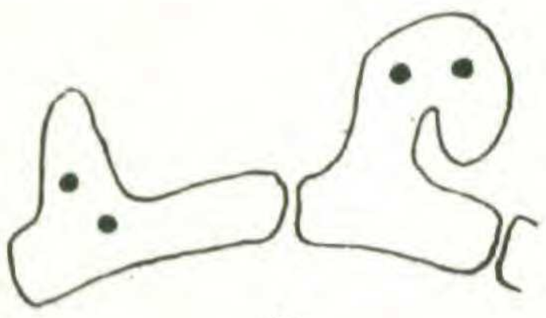

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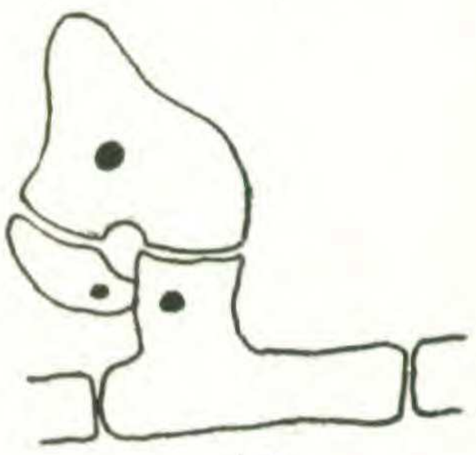

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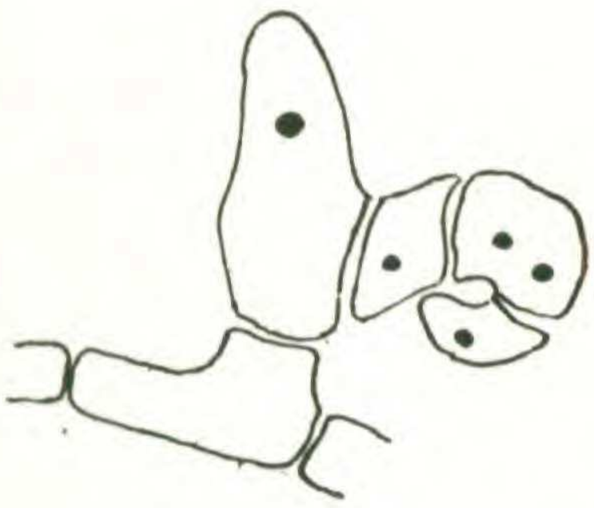

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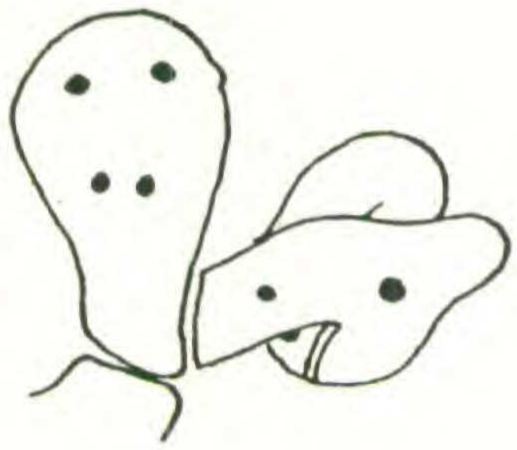

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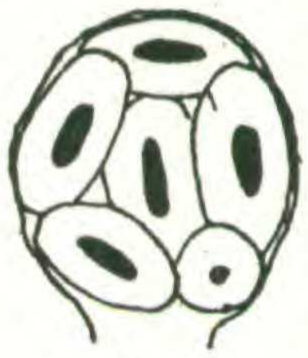

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Text-fig. 1. Male copulation branch. Fig. 2. Female branch. Fig. 3. The two branches before plasmogamy, and fig. 4, after plasmogamy. Figs. 5 and 6. Septa appearing after nuclei pair in the female branch. Fig. 7. Croziers forming and nuclei migrating into one. Fig. 8. Crozier after nuclei divide and septa are formed. Fig. 9. Beginning of first ascus after fusion of nuclei. Fig. 10. Another crozier forming from fusion of nucleus in tip and stem of crook; note also growth of first ascus. Fig. 11. Third crozier forming from the second, ascus of the second developing, and first divisions of nuclei in first ascus. Fig. 12. Mature ascus showing 6 of the 8 ascospores.

('03) for *A. candidus*. She found that both copulation branches were multinucleate, while De Lamater, working with the species related to *A. aureus*, maintained that they were uninucleate. Both authors agree that a septum separates each copulation branch from the main hypha before fusion of the branches. De Lamater claims that fusion of the two nuclei may occur now or at a much later time, while Dale makes no observations on nuclear fusion.

Formation of croziers.—Soon after fusion of the two copulation branches and the pairing of the nuclei, bulges appear at more or less regular intervals on the outside of the copulation branch, one to each cell (pl. 4, figs. 34-36). These develop into croziers (fig. 37). The two nuclei migrate into the crozier hook and divide in the usual manner (text-fig. 7). Two septa then appear, one cutting off the tip and the other separating the crozier from the female copulation branch (pl. 4, fig. 38, text-fig. 8). The crozier now contains one nucleus at the tip, two at the middle, and one in the main cell. A bulge appears on the outside of the cell containing two nuclei. The nuclei fuse and move into this extension of protoplasm which is the beginning of the ascus (text-fig. 9).

Each cell of the female branch produces more than one crozier in much the same manner as described by Claussen ('12) for *Pyronema confluens*. As the two nuclei in the bend of the hook are fusing the tip of the hook comes in contact with the base and plasmogamy occurs (text-figs. 9, 10). After the fusion of these cells, a crozier is formed into which the nuclei move and divide. Another ascus is now formed, and the process is repeated (text-fig. 11).

De Lamater was the first to describe the formation of croziers in any species of *Arachniotus*. Upon referring to Dale's paper ('03), he found that she had noted the occurrence of "short thick hyphae, which branch repeatedly, and form around the coil a dense mat of ascogenous hyphae." From her illustrations, the "short thick hyphae" are evidently croziers. As croziers occur in *A. trisporus*, it is probable that they are characteristic of this genus.

Ascus formation.—The young uninucleate ascus at first may grow more in length than in width, or it may grow uniformly on all sides. In any case a vacuole appears when the ascus is quite small and enlarges as the ascus does. When almost mature the nucleus, which is situated at the end of the ascus, divides. One daughter nucleus usually migrates to the end of the ascus and the other toward the pedicel. The two nuclei divide perpendicular to the axis of the first division; then these four divide, producing eight nuclei. Cell walls soon appear cutting each nucleus off from the others and forming the ascospores. The vacuole begins to disappear about the time of the second division and is soon lost. The ascus wall is very thin and after the formation of the spores gradually disappears. However, the spores usually adhere together for a long time (pl. 5, fig. 2). The formation of the ascus, particularly as regards the vacuole and the division of the nucleus, according to Dale ('03) and De Lamater ('37), corresponds to that found in *A. trisporus*.

CULTURAL REQUIREMENTS

Arachniotus trisporus grows well upon most liquid or solid media employed for the cultivation of fungi. The solid media found to be successful were: Sabouraud's, potato-dextrose, oat-meal, prune, and Vailionis' agar, as well as potato slants. The species also grew upon corn-meal agar, nutrient gelatin (which it did not liquefy), moist sterile feathers, but not as well on these three media as on those first listed. The most successful liquid media were composed of 1 per cent Bacto-peptone and 2 per cent d-glucose or sucrose. Some growth was also produced on 1 per cent Bacto-peptone and 2 per cent lactose medium.

The following is a "time table" of the development of this organism from the time of inoculation as it occurred upon Sabouraud's, potato-dextrose, and oat-meal agar media. It was used as a standard to compare the development of the organism upon other media.

Germination of spores	36–44 hrs.
Production of chlamydospores	42 hrs.
Production of conidia	45 hrs.
Production of copulation branches	66–82 hrs.
Copulation	69–100 hrs.
Formation of croziers	88–124 hrs.
Appearance of asci	104–124 hrs.
Mature ascospores	124–154 hrs.

A series of experiments was carried on to determine the effect of various percentages of carbohydrate and peptone upon the growth and structures produced. As a control, the regular 4 per cent d-glucose Sabouraud's agar was employed. The variations with 2 per cent agar were in each case as follows:

"A" medium—2% d-glucose, 1% peptone.
"B" medium—1% d-glucose, 1% peptone.
"C" medium—½% d-glucose, 1% peptone.
"D" medium—2% d-glucose, ½% peptone.
"E" medium—½% d-glucose, .2% peptone.

Slides were made from each of these cultures at 51, 54, 57, 60, 63, 66, 69, 81, 99, 123, and 144 hours after inoculation with lacto-phenol. No variation occurred in structure and amount of chlamydospores, copulation branches, and ascospores which might not occur in the same culture in the same time. The only variation was in Sabouraud's and "A" medium, which at 51 hours had fewer conidia than the others. From these results, it can be concluded that different percentages of d-glucose and peptone had little or no effect upon the size, form, and time of production of the various structures of this organism.

The same percentages of d-glucose and peptone were again employed, without agar, in a liquid medium. The results here are not very reliable because it is difficult to secure a representative portion of one colony in a flask which will correspond to another in another flask. This becomes more and more difficult as the colony increases in size. In making slides as before it was found that at 46 hours, chlamydospores are always produced before conidia. Copulation branches are not produced until at least 96 hours. Croziers appeared at about 144 hours, and mature ascospores were not seen until 200 or more hours. Summarizing these results, it can be said that broth cultures of *A. trisporus* generally

develop more slowly than agar cultures, and also that, for study, agar cultures are much more adaptable than broth cultures.

Since the cultures developed with equal rapidity regardless of the concentration of nutrients, the organism was inoculated on agar containing only d-glucose and on agar containing only Bacto-peptone. The chlamydospores on the d-glucose medium were at first (51 hours) much smaller than usual, being 4.4 to 5.8 μ in diameter. By 67 hours they had become thick-walled, which is also unusual. The ordinary swollen hyphae were evident, but some of the regular hyphae were greatly enlarged. Conidia were as usual. Copulation branches appeared at 90 hours and mature ascospores at 144, which is not unusual. On the peptone media an abundance of conidia was produced throughout the time the slides were made. Chlamydospores were produced but always on very short branches. At 114 hours copulation was observed, but the next two slides, 138 and 152 hours, showed no sexual reproduction at all. It is evident that more work is needed on this problem before any very definite conclusions can be reached. A combination of the two is necessary for normal growth.

Experiments were run to determine the optimum pH. D-glucose, sucrose, and lactose broths were used, and each medium adjusted to pH 6.4, 7.0, and 7.6 as nearly as possible. After the organism had reached maturity, growth was recorded and the pH determined. It was found that growth was good at pH 6.4 and 7.0, and that usually the pH was changed to 6.0 or below. Growth was usually poor at 7.6, and no change of pH was observed.

Upon incubating 18 Petri-dish cultures of *A. trisporus* on Sabouraud's agar, 6 at 24° C., 6 at 30°, and 6 at 35°, the optimum temperature for colony growth was found to be be between 30° and 35° C. Cultures inoculated at the same time were again placed in the 30° C. incubator, which ranged from 29° to 31°, and the 35° C. incubator ranging from 33° to 37°. Measured over a period of 70 hours, growth at the lower temperature was spreading and flat, while at the higher temperature an abundance of aerial mycelium as well as concentric rings resulted. Those cultures at 30° C. were 0.4 cm. larger than those at 35° C. It can be concluded, then, that the optimum temperature is between 30° and 35° C., most likely 33° C.

PATHOGENICITY

The writer, upon accidentally pricking her finger with a contaminated needle, found that after 48 hours a small vesicle appeared. To confirm the suspicion that this was caused by *A. trisporus*, with aseptic precautions she inoculated a portion of the skin of the arm with spores of this organism. After a period of 48 hours it was evident that the fungus was maintaining itself satisfactorily enough to exist but not to grow to any extent. A small raised slightly inflamed spot on the arm was the only indication of the organism. At the end of three weeks, the lesion was opened aseptically, and some of the tissue placed upon a sterile Sabouraud's agar plate. Within 48 hours, the organism germinated and

grew as usual. The experimental lesions healed spontaneously in about a week. This is the first record of the pathogenicity of any species of *Arachniotus*, to the writer's knowledge. It is interesting to note that it is possible for this fungus to live pathogenetically, and that it certainly causes no serious harm.

IDENTIFICATION

There is no doubt that the species in question is a member of the genus *Arachniotus*. It possesses the characteristics which Schroeter ('93) lists for the genus, namely, a globose fruit-body of interwoven hyphae resembling a spider web, and spherical or ellipsoidal spores, the membranes of which are either hyaline, golden, or red. It cannot be placed in closely related genera, such as *Gymnoascus*, because of the absence of spines on the hyphae of the peridium, nor in *Amauroascus*, because of the brown peridium, nor in *Ctenomyces*, because of the well-developed peridium and the comb-like appendages. Plate 5, fig. 2, illustrates the loose non-appendaged peridium of *A. trisporus*.

In determining the true species, the characteristics of all species known will be listed. They are as follows:

ARACHNIOTUS RUBER Schroet. in Cohn, Krypt. Fl. Schles. **3**^{2}:211. 1893.

Gymnoascus ruber Van Tiegh., Bull. Soc. Bot. France **24**:159. 1877.

Fruit-body orange-red or red, 0.5 mm. in diameter at the most; ascospores orange-red or red, 4.5 x 3.5 μ; only 6 to 20 asci per perithecium, fewer than in other species. Isolated from dung of dog, rat and goat at Breslau; from cat's dung, Aburi, Gold Coast.

ARACHNIOTUS CANDIDUS Schroet. in Cohn, Krypt. Fl. Schles. **3**^{2}:210. 1893.

Gymnoascus candidus Eidam, Schles. Ges. Vaterl. Kultur, Ber. Bot. Sect. 160-165. 1886.

Fruit-body snow-white, globose, 0.5–2 mm. in diam.; ascospores hyaline, ellipsoidal, smooth, 3.5 x 3 μ; conidia pyriform, in chains (fide Schroeter, none reported by Eidam, probably the oidia of Dale and aleurospores of Nannizzi). Isolated from boiled rice, owl's dung in Brieg, old nest of wild bee and dung of common roe at Kew, England.

ARACHNIOTUS AUREUS Schroet. in Cohn, Krypt. Fl. Schles. **3**^{2}:210. 1893.

Gymnoascus aureus Eidam, Schles. Ges. Vaterl. Kultur, Ber. Bot. Sect. 160-165. 1886.

Fruit-body golden-yellow, 0.5–1 mm. in diam.; with fine hyphal spirals in the peridium; ascospores golden-yellow, spherical to ellipsoidal, spiny, 3.5–4.0 μ in diam.; conidia not reported by Schroeter, aleurospores by Nannizzi. Isolated from decaying vegetables.

ARACHNIOTUS CITRINUS Massee & Salm., Ann. Bot. **16**:62. 1902.

Fruit-body lemon-yellow; ascospores ovoid to subglobose, smooth, 4–5 x 2.5–3.5 μ; conidia not reported. Isolated from dung of giant kangaroo, Kew, England.

ARACHNIOTUS TRACHYSPERMUS Shear, Science, N. S. **16**:138. 1902.

Fruit-body white; ascospores faintly greenish-yellow, echinulate, 3.25–4.0 x

2.0–2.5 μ; conidia resembling those of *Penicillium*. Isolated from diseased cranberries.

ARACHNIOTUS TRISPORUS Hotson, Mycologia **28**:497-500. 1936.

Fruit-body white, turning yellowish with age, 160–326 μ in diam.; asci ellipsoidal to spherical, 7–9 x 10–11 μ; ascospores hyaline, ellipsoidal, smooth, 3.5 x 5.5 μ; conidia hyaline, ellipsoidal, 3.5–4.5 x 4.5–5.5 μ; chlamydospores subspherical to pyriform, 6–7 x 7–11 μ. Isolated from contaminated milk.

GYMNOASCUS SUDANS Vailionis, Vyt. Didžiojo Univ. Mat.-Gamtos. Fak. Darbai **11**:119. 1936.

Fruit-body white; asci spherical to pyriform, 13.2 μ in diam.; ascospores hyaline, ellipsoidal, smooth, 8.8 x 6.6 μ; conidia hyaline, ellipsoidal, 5.3 x 4.4 μ; chlamydospores spherical to pyriform, hyaline, 8.8 μ. Isolated from a nutrient solution in which birch twigs were being cultivated.

GYMNOASCUS SUDANS Vailionis (description based on subculture of Vailionis' original culture).

Fruit-body white turning to cartridge-buff upon aging, size variable; asci ellipsoidal to spherical, 7.3–7.5 x 10.2–10.6 μ; ascospores hyaline, ellipsoid, smooth, 3.1–3.3 x 4.3–4.6 μ; conidia hyaline to cartridge-buff, pyriform, 1.5–1.9 x 2.2–3.1 μ; chlamydospores cartridge-buff, pyriform, 6–8 x 8–10 μ.

Of the above species, it is possible to place Vailionis' organism under *Arachniotus trisporus*, as has been previously done, or under *A. candidus*. It being impossible to obtain a culture of *A. candidus* it is necessary to rely strictly upon the literature, which is certainly not complete and quite contradictory. Since the size of the ascospores of that species differs slightly from that of *A. trisporus*, the description of conidia conflicting, and since no mention is made of chlamydospores, exudate, or the color upon aging, the writer must conclude that to call this species *A. trisporus* is justifiable. Furthermore, it being impossible at the present time to determine the month in which Vailionis published his description, and since his organism is already known as *A. trisporus*, we believe that it should continue to be known under that name.

Since Vailionis discovered his species in Lithuania and Hotson his in the state of Washington, it was thought that some difference must occur between the two cultures. However, after a microscopical study of the two, their morphological structures were found to be identical in size and relative abundance. Colony characteristics, however, were slightly different (pl. 6). The cultures were then designated as Vailionis' strain and Hotson's strain.

After making a thorough study of the culture identified by Nannizzi as *A. aureus*, obtained from Baarn through De Lamater, the writer concluded that this organism was of the same species but a different strain from the other two. Its colony characteristics (pl. 6) are different from the others, but the color of the mycelium, the exudate, and its morphological characters are identical. For these reasons the writer designates this culture as *A. trisporus*, Nannizzi's strain.

SYSTEMATIC POSITION OF ARACHNIOTUS

Arachniotus occupies a position between the Gymnoascaceae and the Aspergilliaceae, because it has a loosely woven peridium as found in the former, and conidia resembling very strongly those found in the latter. It should be considered as one of the lowest members of the family Gymnoascaceae, since there are only traces of a peridium the hyphae of which are undifferentiated. It is the only known member of this family showing crozier formation and, according to De Lamater, the most primitive fungus having croziers. The presence of conidia in *Arachniotus,* produced from such similar structures as found in *Aspergillus* and *Penicillium* and also in a similar fashion, supplies a strong link to bridge the gap between the Gymnoascaceae and the Aspergilliaceae.

It has long been thought that the Trichophytoneae or the Dermatophytes were imperfect forms of the Gymnoascaceae. (For a review of the literature pertaining to this subject, see Dodge, '35). However, definite proof of this relationship has not as yet been established. Pollacci ('25) stated that when *A. candidus* is cultivated on Pollacci agar perithecia do not develop and that the organism closely resembles *Trichophyton.* Nannizzi ('26) cultivated *T. radiolatum, T. asteroides, T. denticulatum,* and *T. felineum* upon feathers, skin, leather, and bones, and found that they produced pycnidia resembling the ascocarps of *Ctenomyces serratus, Arachniotus candidus, A. aureus,* and *Gymnoascus Reessii,* which he also studied. In this paper he brought out, besides other morphological similarities, the significance of finding the racquet mycelium in *Microsporon* and in *Ctenomyces serratus* and *Myxotrichum uncinatum.* Although the writer was unable to make such a study as Nannizzi did, she believes that the presence of the racquet mycelium in the perfect and imperfect forms is a morphological connection between these two groups. Nannizzi has shown in his figures that *Ctenomyces serratus, Myxotrichum uncinatum, Microsporon lanosum,* and *Trichophyton radiolatum* (to some extent) all possess racquet mycelium. Illustrations in Ota and Langeron ('23) show that *Microsporon Audouini, Megatrichophyton equinum, M. ferrugineum,* and *Favotrichum ochraceum* possess similar mycelium.

From the above, three significant findings should be brought out: First, racquet mycelium occurs almost invariably in all three strains of *A. trisporus.* Second, *A. trisporus* has an optimum growth temperature between 30 and 35° C., a range at which most of the Dermatophytes grow the best. Third, and one of the most important, *A. trisporus* can definitely exist as a parasite. These three facts all point towards the conclusion that the Trichophytoneae are the imperfect forms of the Gymnoascaceae, but unfortunately does not finally establish this theory as a fact.

SUMMARY

1. *Arachniotus trisporus,* besides having regular multinucleate hyphae of a more or less constant width and length, is characterized by a racquet mycelium similar to that found in the Gymnoascaceae and the Trichophytoneae.

2. *A. trisporus* reproduces asexually by multinucleate chlamydospores and uni- or binucleate conidia. The chlamydospores have proved to be the most viable type of spore over a period of years. The conidia are produced in chains from a phialide as in the Aspergilliaceae.

3. In sexual reproduction, two unlike multinucleate copulation branches are produced which, after copulating, fuse. The male nuclei move into the female branch and the nuclei pair. Cell walls appear between each pair, and croziers are formed. The two nuclei in the tip of the crozier fuse and divide three times, producing eight uninucleate ascospores. The ascus wall soon disappears but the ascospores usually remain clumped together as they appeared in the ascus.

4. This species is capable of good growth upon Sabouraud's, potato-dextrose, oat-meal, prune, and Vailionis' agar. Growth in liquid media of the same composition, but without the agar, is good, but the development of the organism is slower. A "time table" of the appearance of the various morphological structures is given. It is found that the optimum pH is near 6, and that the optimum temperature is between 30° and 35° C.

5. *Gymnoascus sudans* Vailionis is identical with *A. trisporus* Hotson.

6. This species is capable of existing as a pathogen.

7. Because of the presence of a loose undifferentiated peridium, *A. trisporus* belongs in the Gymnoascaceae, but, on the other hand, it produces conidia similar to those found in the Aspergilliaceae. It is therefore believed that this organism occupies a position between the two families.

8. The presence of a racquet mycelium in *A. trisporus*, similar to *Ctenomyces* and *Myxotrichum*, and also species in the Trichophytoneae, suggests that the Trichophytoneae are related to the Gymnoascaceae. Besides the presence of a racquet mycelium, the optimum temperature of 30° and 35° C. and the demonstration of the pathogenicity of this species also point in this direction.

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Explanation of Plate

PLATE 1

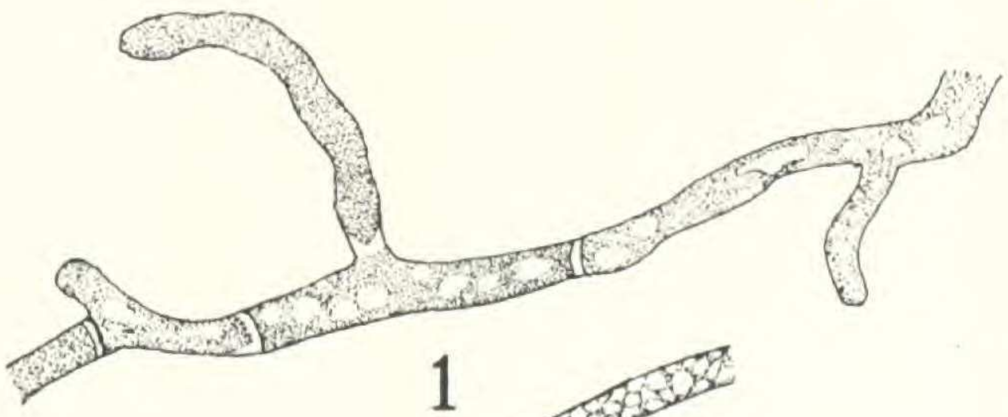
Arachniotus trisporus

Figs. 1–6. Vegetative hyphae from lacto-phenol prepared slides from potato-dextrose agar cultures, showing development of the vacuole from time of inoculation. Width of hyphae shown is 2.5 μ.

Fig. 1, after 51 hours.
Fig. 2, after 60 hours.
Fig. 3, after 69 hours.
Fig. 4, after 81 hours.
Fig. 5, after 99 hours.
Fig. 6, after 144 hours.

Figs. 7–12. Vegetative hyphae from iron-haematoxylin prepared slides showing nuclei and development of the vacuole from time of inoculation.

Figs. 7–9. Hyphae 2.5 μ in width, from 52-hour potato-dextrose agar cultures.
Figs. 10–11. Hyphae 2.5 μ in width from 60-hour Sabouraud's agar culture.
Fig. 12. Hyphae 2.2 μ in width from 81-hour potato-dextrose agar culture.



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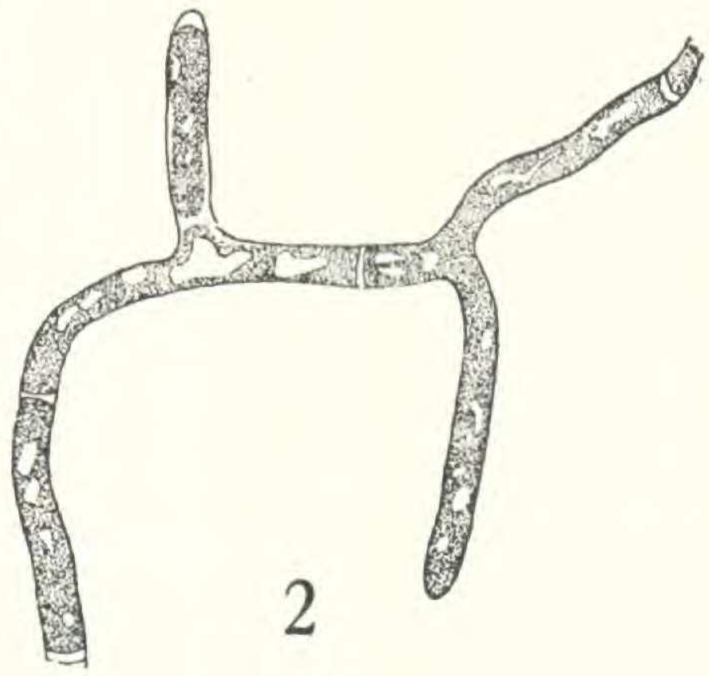

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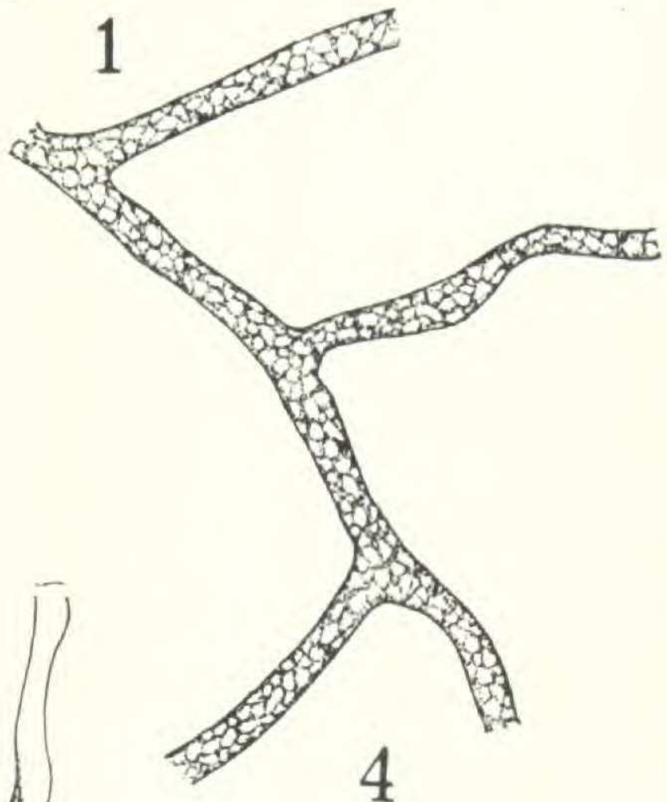

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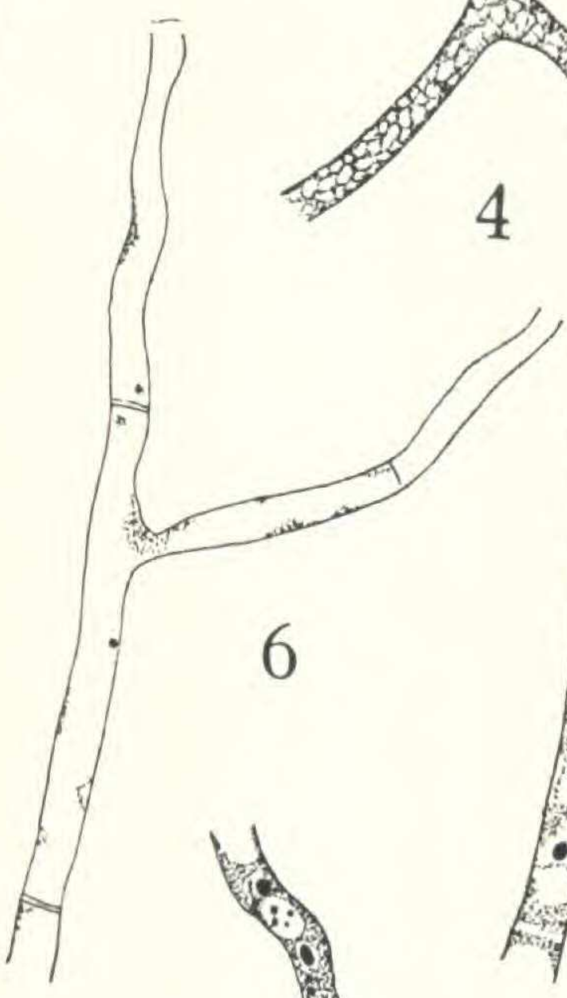

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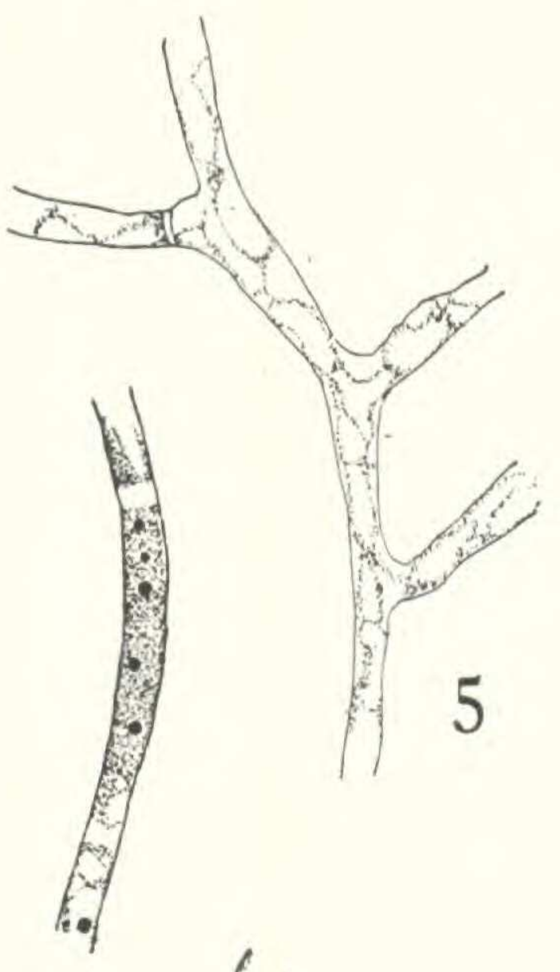

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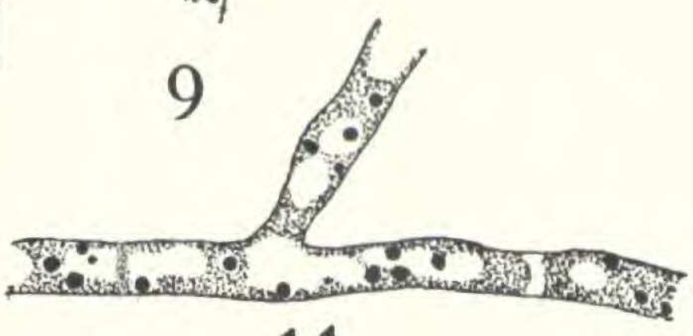

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EXPLANATION OF PLATE

PLATE 2

Arachniotus trisporus

Figs. 13–14. Portion of racquet mycelium at 57 hours taken from lacto-phenol prepared slides made from potato-dextrose agar cultures:

Fig. 13. Cell marked *a* is 10 μ at widest part and 54.5 μ long.

Fig. 14. Cell marked *a* is 15.5 μ at widest part and 43.7 μ long.

Figs. 15–21. Chlamydospores and germinating spores taken from lacto-phenol prepared slides except fig. 20, which is from an iron-haematoxylin preparation.

Fig. 15. From a 51-hour oat-meal agar culture: *a*, 5.6 x 7.5 μ; *b*, 4.4 x 4.4 μ; *c*, 5.0 x 5.6 μ.

Fig. 16. From a 48-hour potato-dextrose culture, showing continuation of hyphae from apical end of spore: spore 10.0 x 11.3 μ.

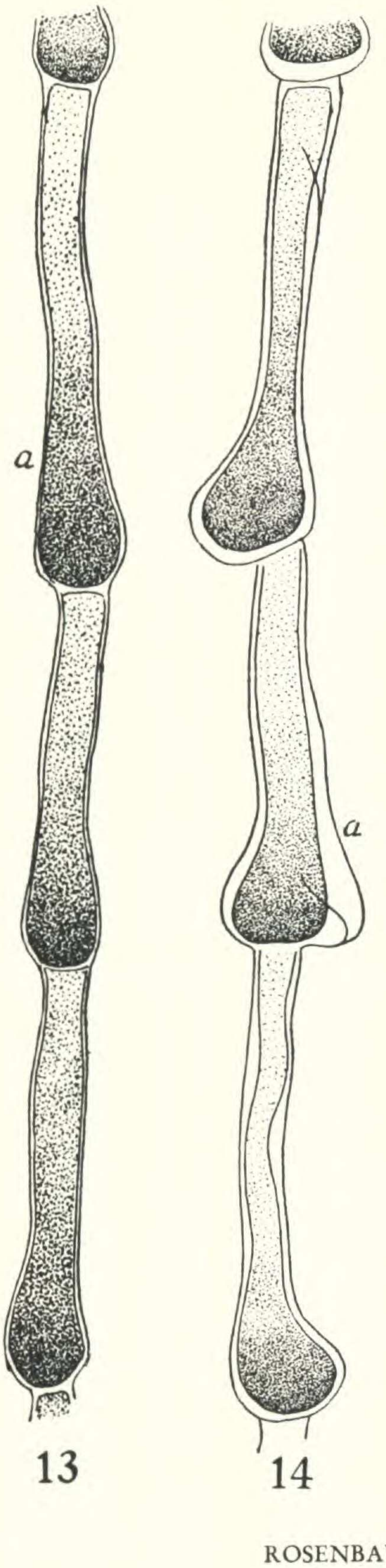
Fig. 17. Sprouting chlamydospore from a 45-hour potato-dextrose culture: spore 8.8 x 10.0 μ; projection 10 μ long.

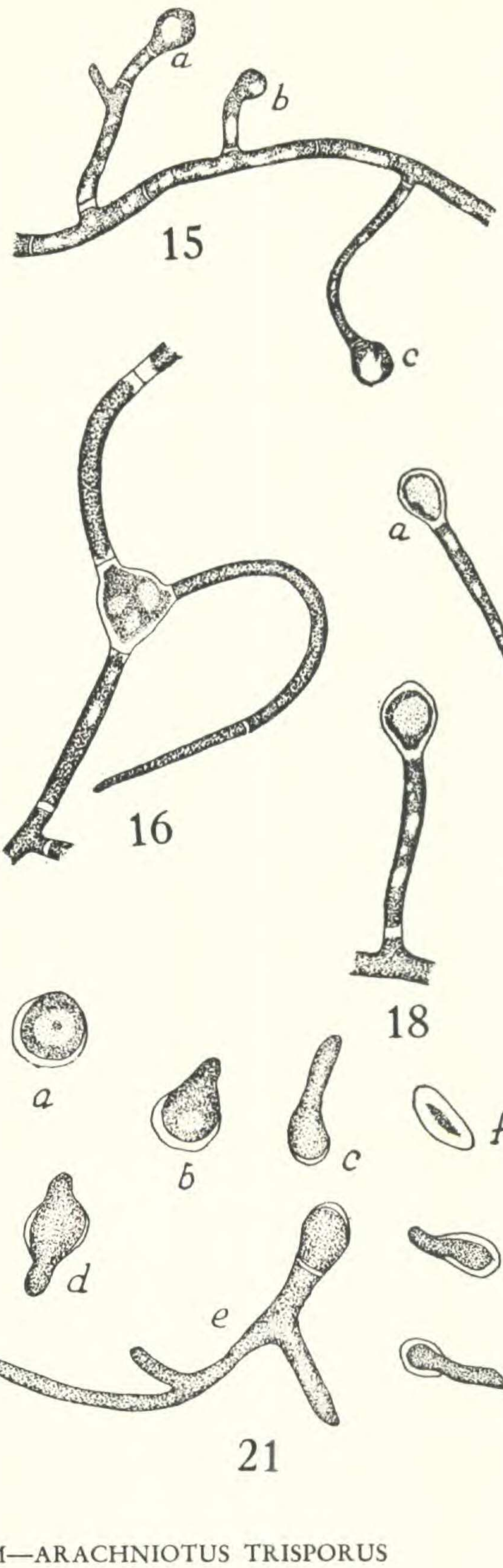
Fig. 18. From a 78-hour oat-meal agar culture; chlamydospore 8.8 x 9.4 μ.

Fig. 19. From a 94-hour Sabouraud's agar culture: *a*, 8.1 x 5.6 μ; *b*, 6.2 x 6.2 μ.

Fig. 20. Showing nuclei from a 46-hour culture.

Fig. 21. *a*, *b*, *c*, *d*, and *e*, germinating chlamydospores; *f*, *g*, *h*, *i*, germinating ascospores.





ROSENBAUM—ARACHNIOTUS TRISPORUS

EXPLANATION OF PLATE

PLATE 3

Arachniotus trisporus

Illustrations of conidia, all taken from lacto-phenol prepared slides except fig. 26.

Fig. 22. Immature conidia taken from a 51-hour culture; hypha 1.3 μ in width; terminal spore 2.5 μ in diameter.

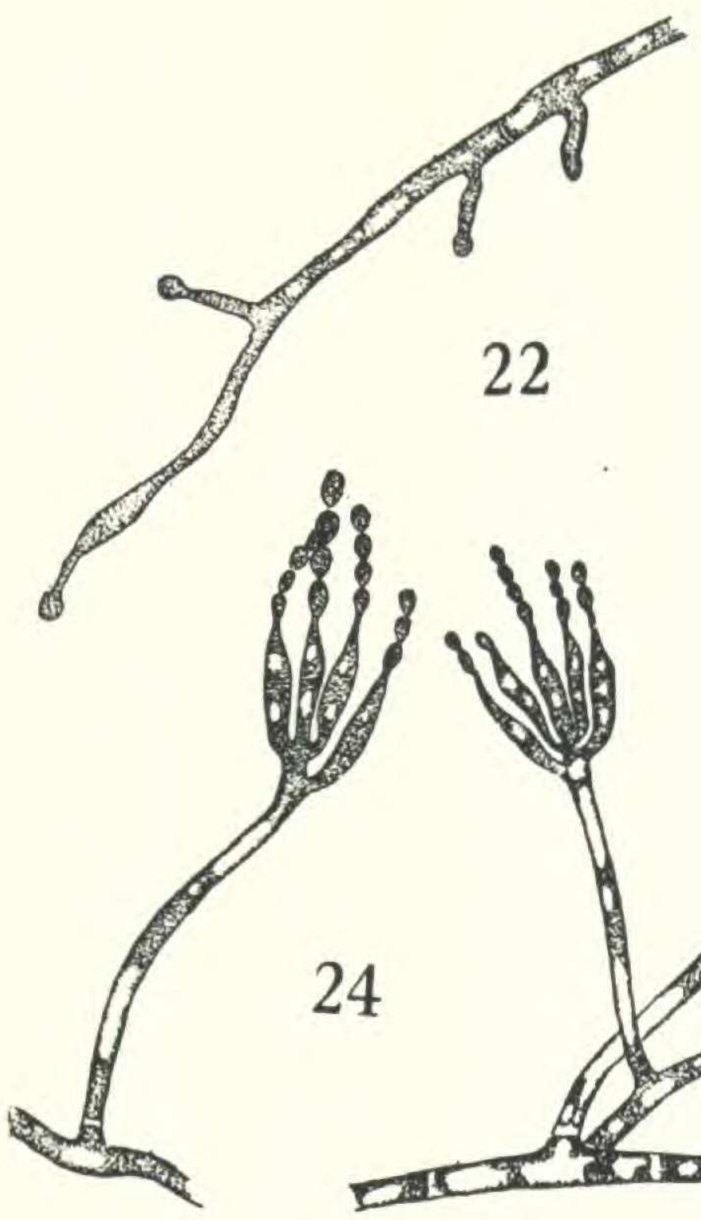
Fig. 23. Branch of conidia taken from a 54-hour oat-meal agar culture.

Fig. 24. An unique type of conidium produced on oat-meal agar between 61 and 69 hours.

Fig. 25. Phialides, after the production of conidia, continuing growth as hyphae; taken from an 81-hour culture.

Fig. 26. Conidia stained with iron-haematoxylin showing some with one, and some with two nuclei.

Fig. 27. Conidia from a 54-hour culture.



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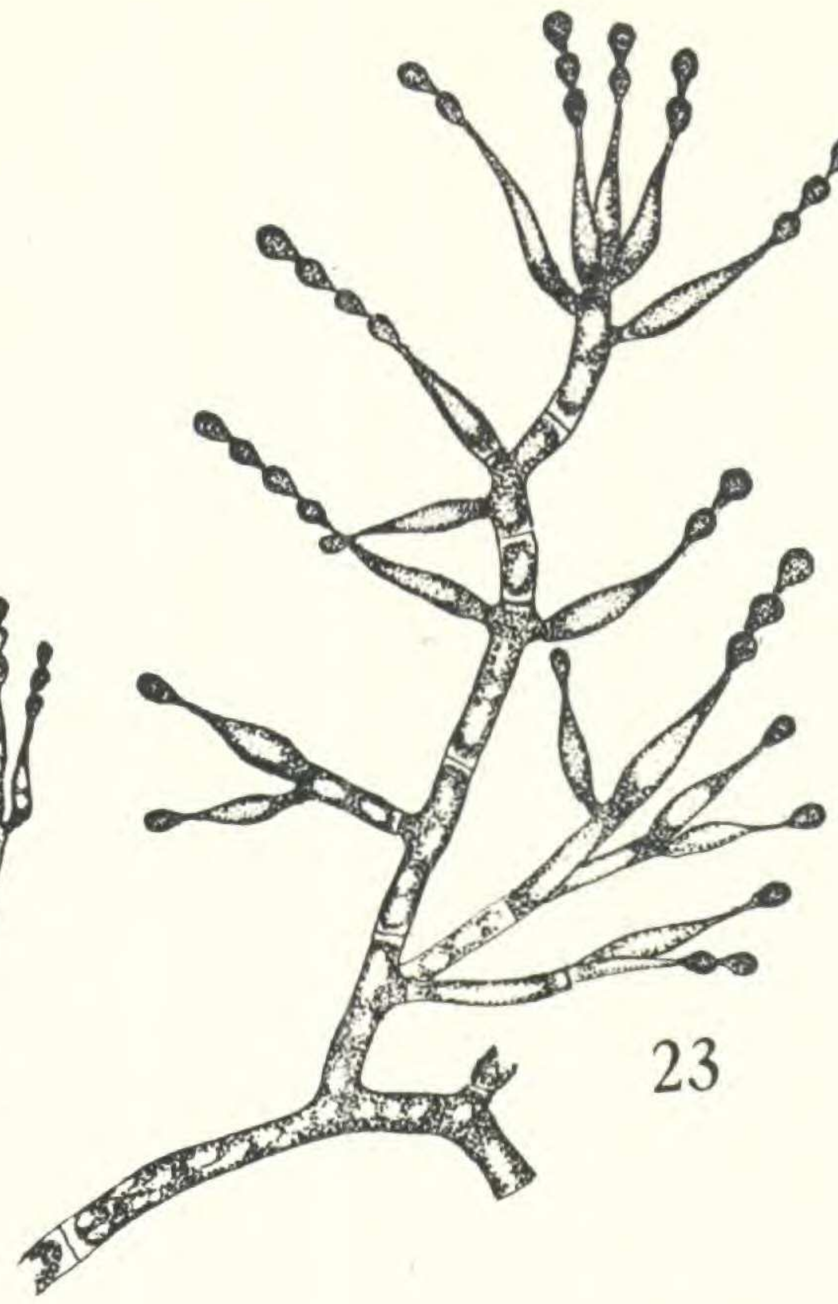

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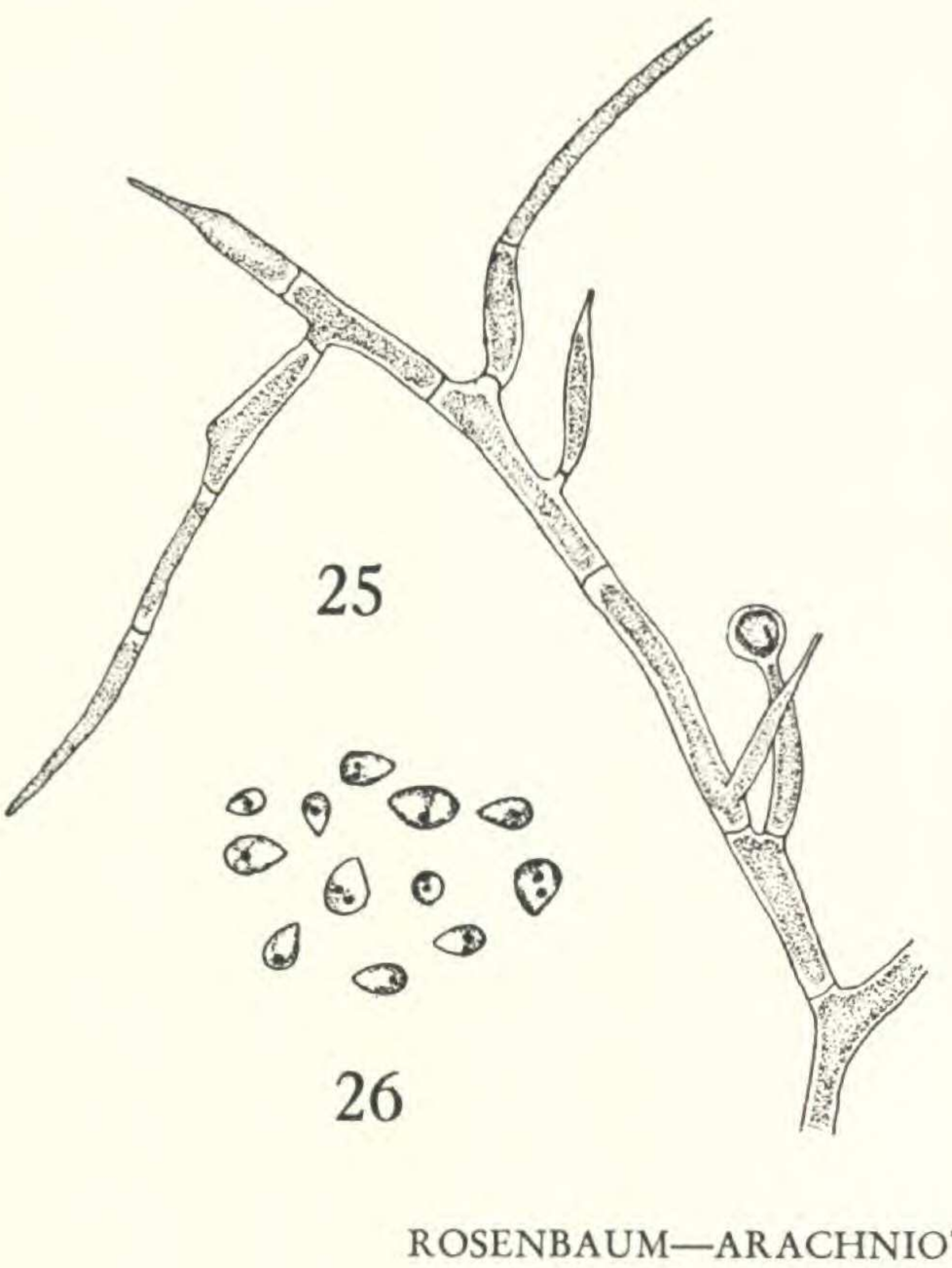

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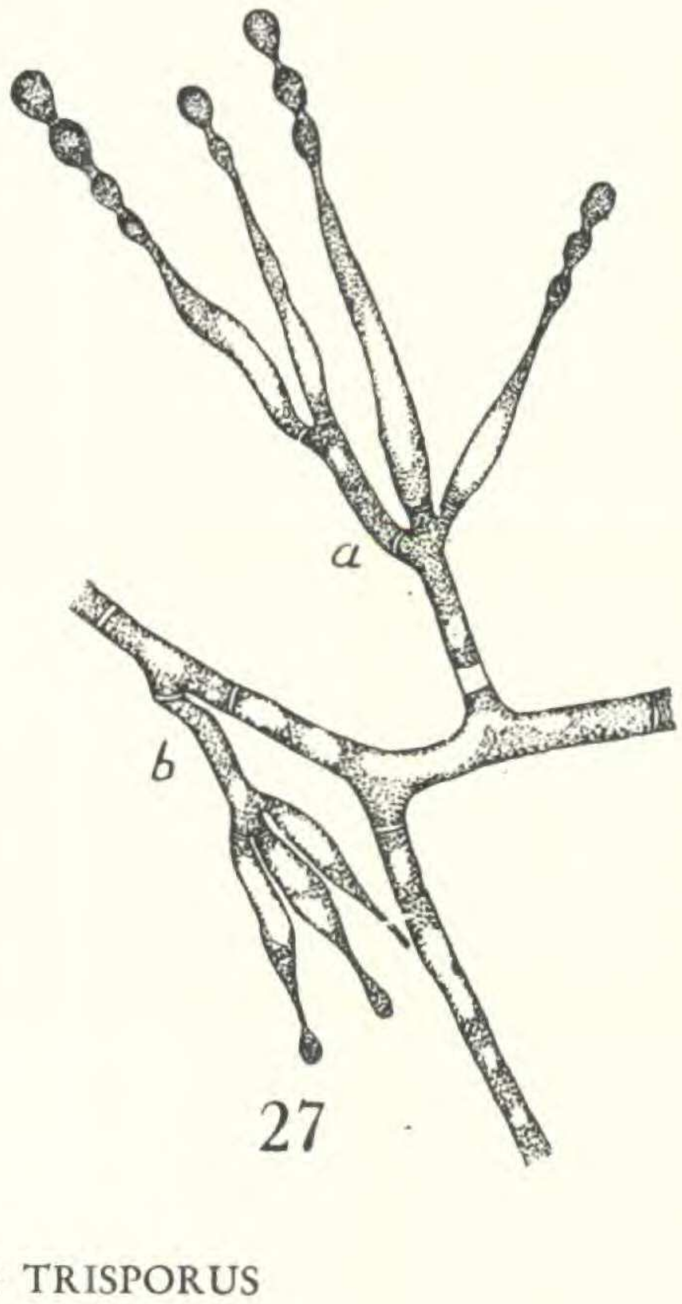

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Explanation of Plate

PLATE 4

Arachniotus trisporus

Illustrating copulation branches, copulation, and formation of croziers, taken from lacto-phenol prepared slides.

Fig. 28. Copulation branches from a 98-hour potato-dextrose agar culture; *a*, *c*, *e*, and *g* are male, while *b*, *d*, *f*, and *h* are female copulation branches.

Fig. 29. Female copulation branch drawing toward male.

Fig. 30. Female branch coiling around the male; meanwhile the tip of each branch has been cut off by a septum.

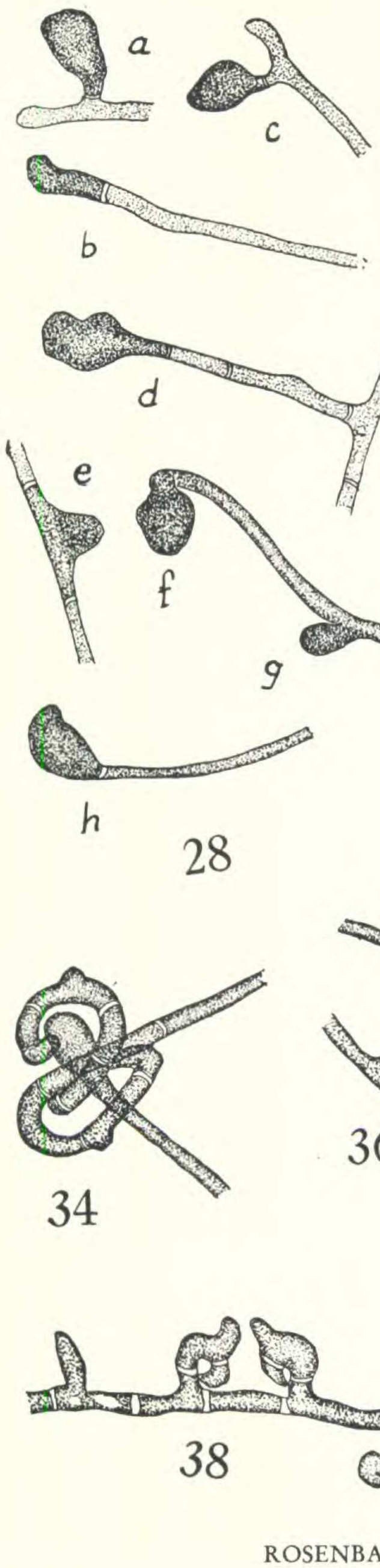
Fig. 31. Copulation of the male and female branches, showing the septa that have appeared before the first septum in the female copulation branch.

Fig. 32. Illustrating the attraction of a female branch from another hypha to the male, and repulsion of the adjacent female branch.

Fig. 33. Septa appearing in the female branch after plasmogamy has taken place.

Figs. 34–37. Various steps in the formation of croziers after septa have divided the female branch into several cells.

Fig. 38. Portion of original female copulation branch showing mature croziers which have begun to form asci.



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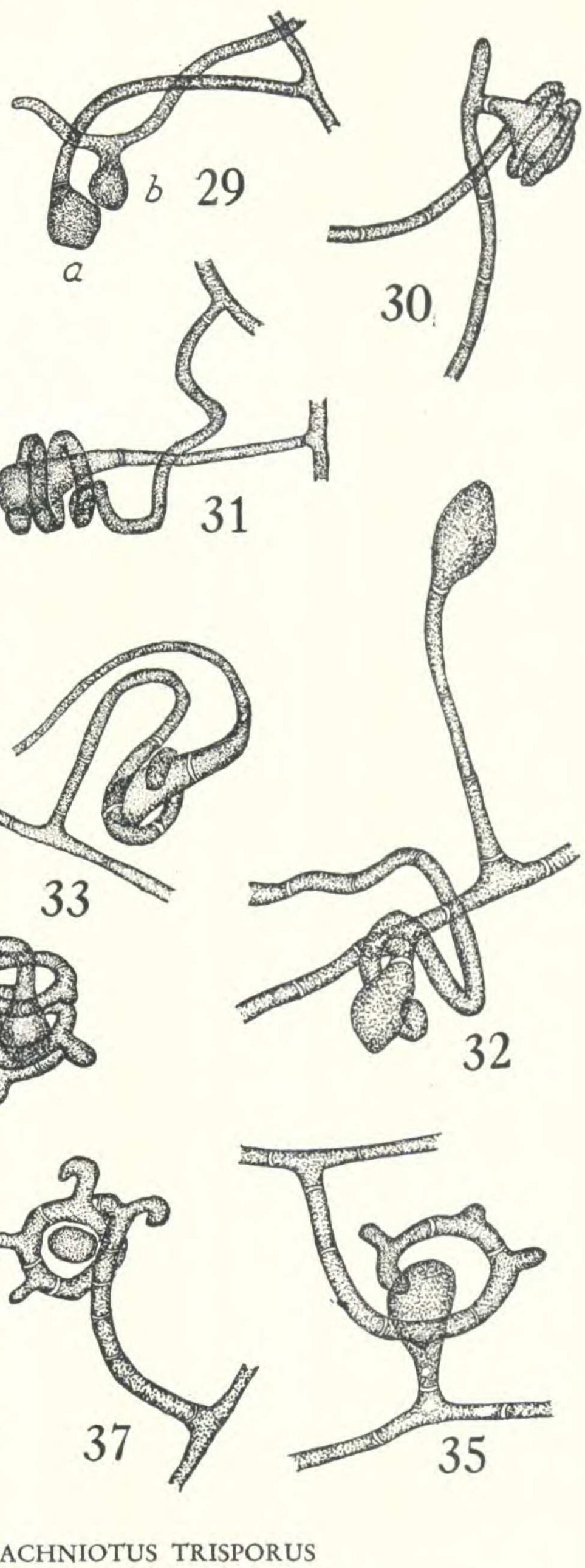


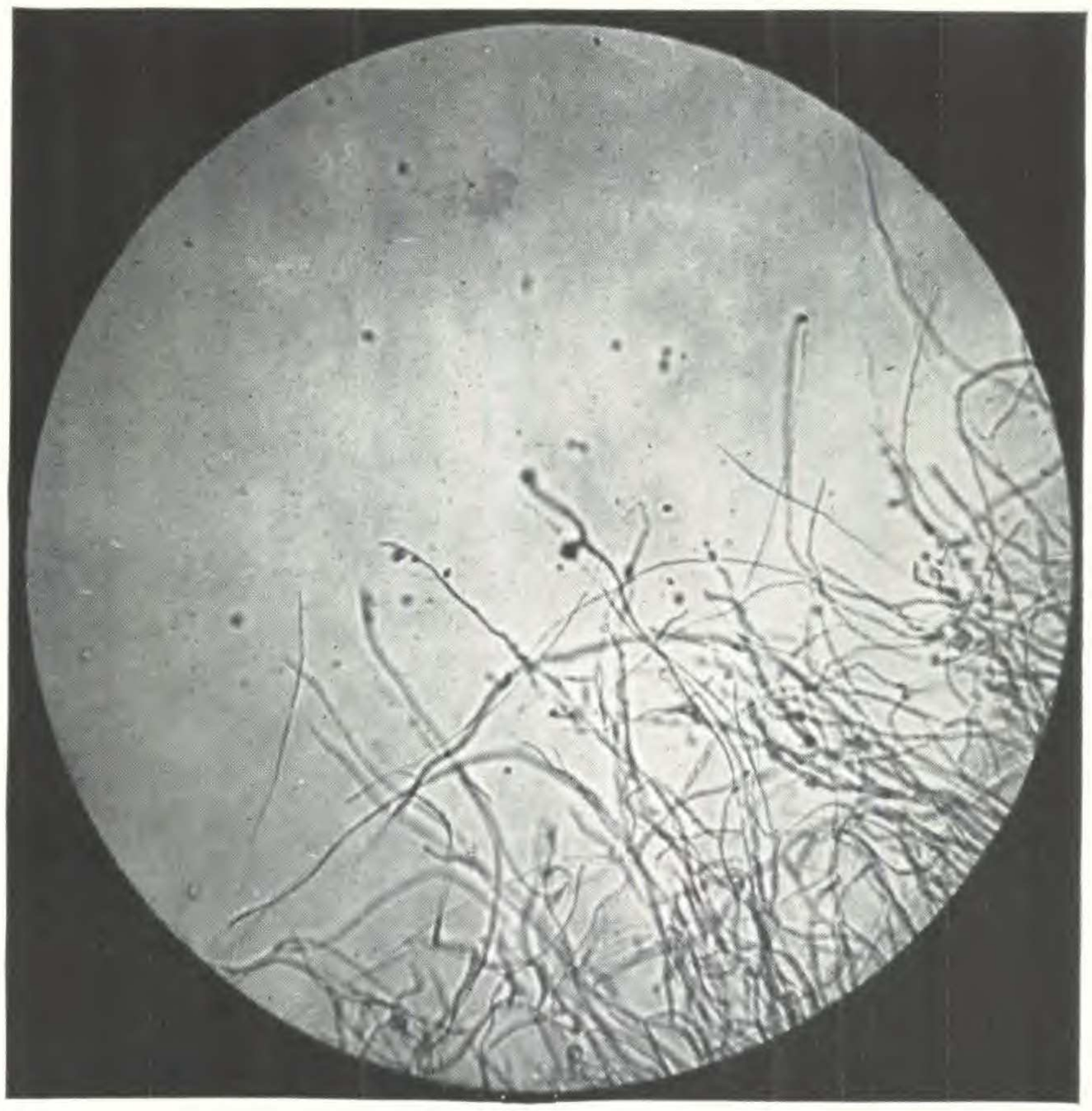
EXPLANATION OF PLATE

PLATE 5

Arachniotus trisporus

Fig. 1. Photomicrograph of vegetative hyphae, showing copulation branches at the extreme ends (x approx. 360).

Fig. 2. Photomicrograph of a fruit-body, showing asci and ascospores (x approx. 745).



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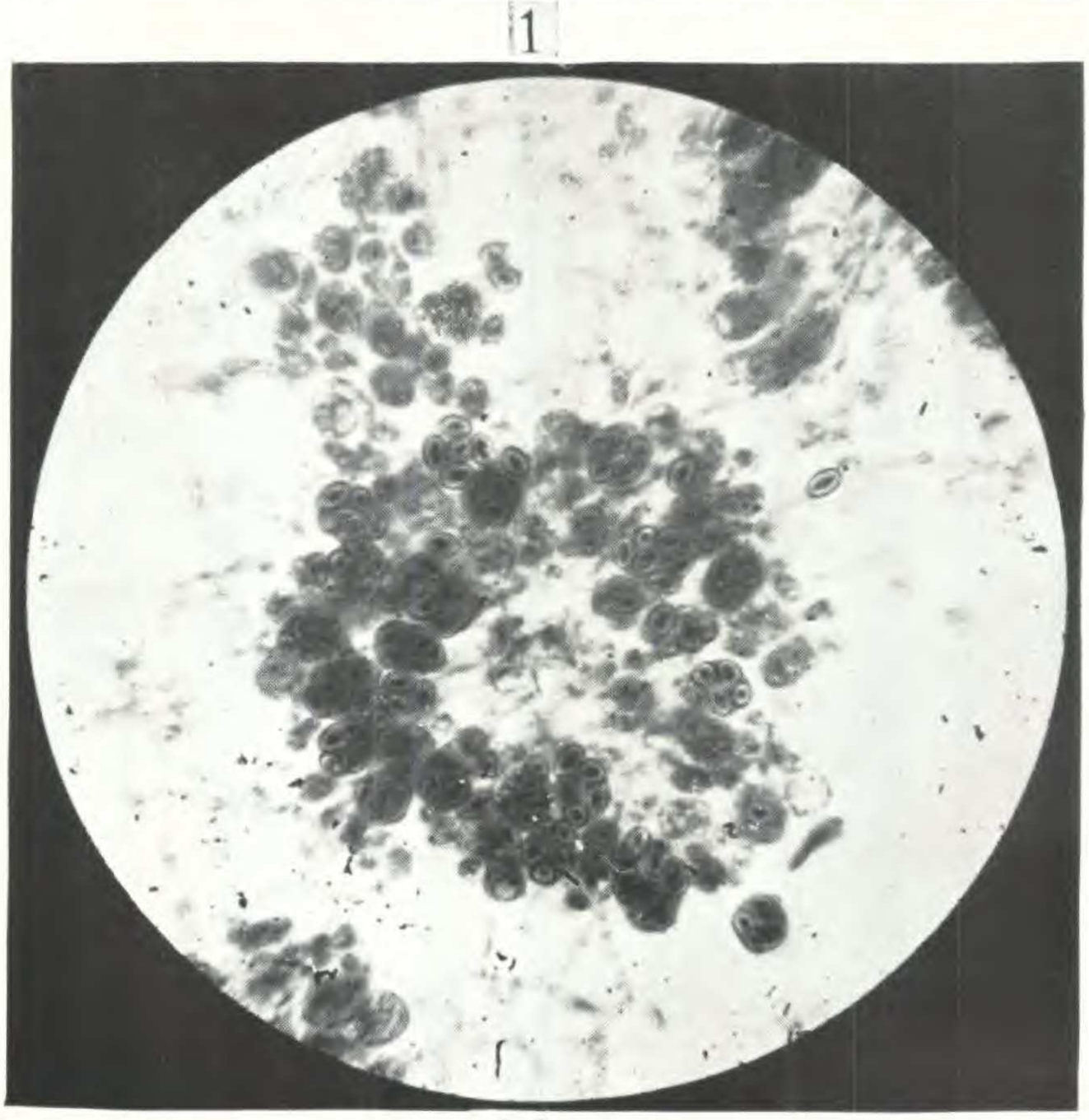

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ROSENBAUM—ARACHNIOTUS TRISPORUS

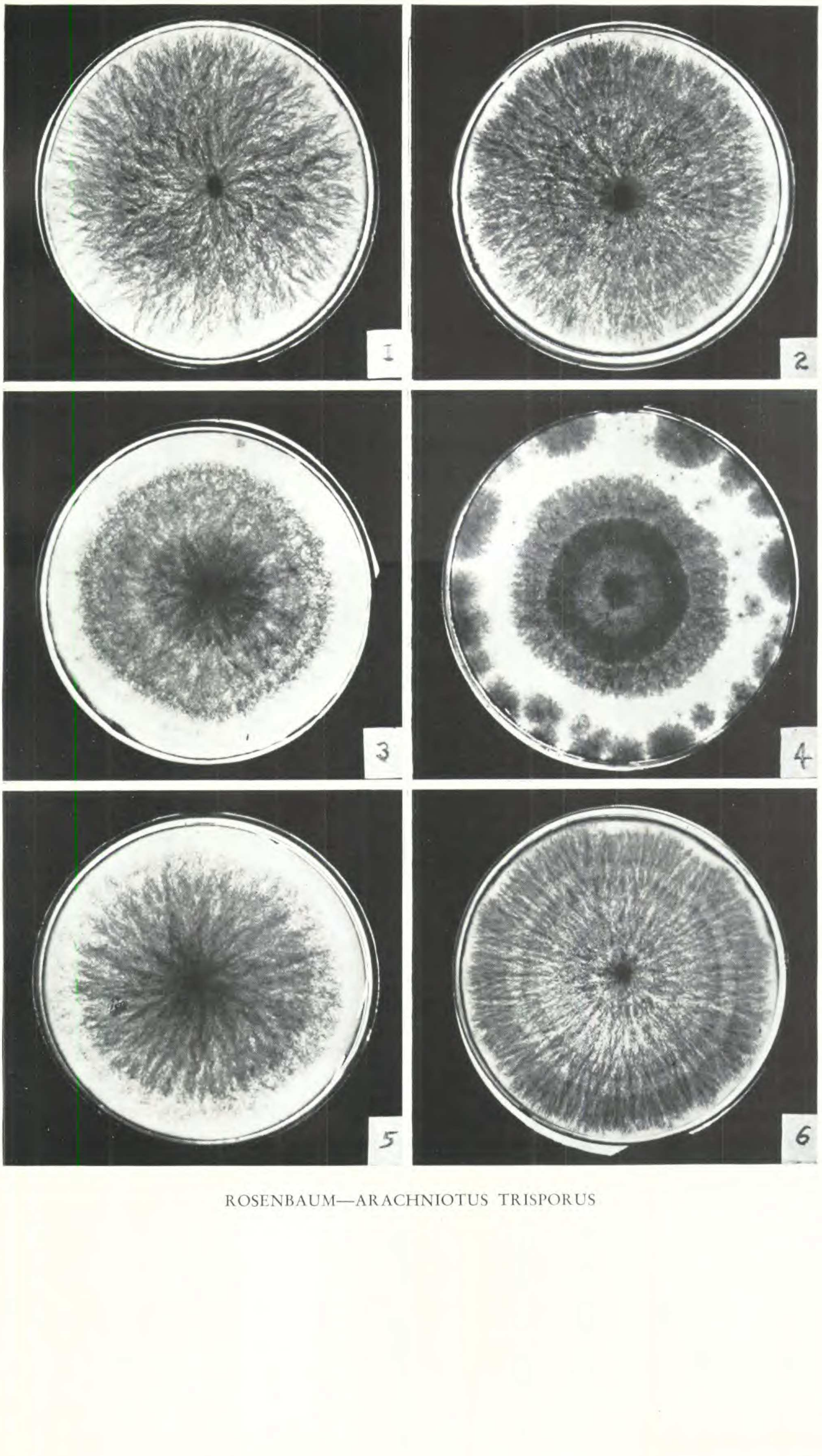
Explanation of Plate

PLATE 6

Arachniotus trisporus

Photographs of Petri-dish cultures of the three strains grown on Sabouraud's agar. Figs. 1, 3, and 5 were incubated at approximately 30° C., and figs. 2, 4, and 6, at approximately 35° C. Note the formation of rings when grown at 35° C.

Figs. 1 and 2. Vailionis' strain.
Figs. 3 and 4. Nannizzi's strain.
Figs. 5 and 6. Hotson's strain.



ROSENBAUM—ARACHNIOTUS TRISPORUS